EXHIBIT 82

Presenilin 1 interaction with cytoskeleton and association with actin filaments

Michael Sych, Henrike Hartmann, Barbara Steiner and Walter E. Mueller[CA]

Department of Pharmacology, Biocenter Niederursel, University of Frankfurt, Marie-Curie-Str. 9, N260, D-60439 Frankfurt, Germany

[CA]Corresponding Author

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Presenilin 1 (PS1) has been shown to interact with microfilament-associated proteins of the filamin family. Here, we investigated a possible association of PS1 with the cytoskeleton. Immunoblotting of detergent-insoluble fractions of rat brain homogenate revealed enrichment of neuron-specific 36 and 14 kDa proteolytic fragments of PS1, whereas 30 and 20 kDa fragments were found in the detergent-soluble fraction. Specific severing of microfilaments with gelsolin in the detergent-insoluble pellet and subsequent centrifugation led to the detection of both actin and PS1 fragments in the supernatant. In addition, *in vitro* translated PS1 cosedimented with actin filaments. Our findings provide biochemical evidence for the association of PS1 fragments with actin filaments. *NeuroReport* 11:3091–3098 © 2000 Lippincott Williams & Wilkins.

Key words: Actin; Alzheimer's disease; Cytoskeleton; Presenilin 1; Subcellular localization

INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is a progressive neurodegenerative disorder of the human brain. Most cases of AD are sporadic. Autosomal dominantly inherited forms contribute to roughly 10% of all AD cases and are generally early in onset. So far, mutations in three genes are known to cause AD, the amyloid precursor protein (APP) gene on chromosome 21 [1], the presenilin 1 (PS1) gene on chromosome 14 [2] and the presenilin 2 (PS2) gene on chromosome 1 [3]. The latter two encode two highly homologous proteins. The large majority of early-onset familial AD cases are caused by mutations in the PS1 gene with more than 50 pathological mutations reported so far. Today, both the contribution of presenilin mutations to the pathomechanism of AD and the physiological function of presenilins are only partially understood, but data provide evidence for roles in APP processing, membrane trafficking or Notch signalling [4,5].

Presenilins were suggested to possess 6–8 transmembrane regions with the *N*-terminus, the *C*-terminus and the large hydrophilic loop directed to the cytosolic face of the membrane [6–8]. Endoproteolytic processing of full-length PS1 is precisely controlled and results in discrete constitutive 30 kDa *N*-terminal and 20 kDa *C*-terminal fragments in the adult rat. Additional alternative proteolytic cleavage of PS1, which is restricted to the brain, generates discrete 36 kDa *N*-terminal and 14 kDa *C*-terminal fragments. Alternative cleavage products were shown to be neuron specific and gradually increase in the course of neuronal differentiation. In human tissue, constitutive PS1 fragments similar to those found in peripheral rat tissue are present, whereas alternative *N*-terminal PS1 fragments are more heterogeneous and migrate between 30 and 40 kDa [9,10]. Caspase cleavage presents another possibility for PS1 fragmentation and generates PS1 species with apparent mol. wts comparable to alternative PS1 fragments in human and rodent brain, but these do not show the heterogeneity displayed by the alternative fragments found in human brain [11].

Presenilin 1 is ubiquitously expressed, and highest levels of PS1 mRNA and protein are found in neurons [12–14]. Immunochemical studies of PS1 transfected non-neuronal cells and undifferentiated neuroblastoma cells show high expression levels of PS1 in the endoplasmic reticulum (ER) and lower levels in the Golgi complex. In differentiated neurons, PS1 co-localizes with BiP, a marker for the rough ER, in the somatodendritic compartment but PS1 also appears in neuritic processes showing no staining for BiP [9,10,15,16].

The existence of PS1 in non-ER and non-Golgi compartments and also the interaction of PS1 with intraneuronal neurofibrillary tangles (NFTs) and actin-associated proteins such as filamin and FH-1 [16,17] encouraged us to further elucidate the potential association of PS1 with the cytoskeleton. For this purpose, we investigated endogenous levels of PS1 via a biochemical approach.

MATERIALS AND METHODS

Tissue preparation: Male Wistar rats were deeply anesthetized with CO_2. Brains were immediately removed and stored at −80°C. Before use, cerebelli were cut off and discarded.

Antibodies: Rabbit antiserum 231-f was raised against a synthetic peptide corresponding to residues 2–20 of PS1 [2] conjugated to keyhole limpet hemocyanin. Both antisera R27 and R28 were raised in rabbits with a GST fusion protein encompassing residues 263–407 of PS1. All PS1 antibodies used have been characterized previously and their specificity was determined by preabsorption experiments [9,16].

Other antibodies used were monoclonal mouse anti-BiP (SPA-827, StressGen, Hamburg), anti-actin (clone AC-40, Sigma, Deisenhofen) directed against all isoforms of actin, anti-α-tubulin (clone DM 1A, Sigma, Deisenhofen) and anti-NF145 (clone NN-18, Calbiochem, Bad Soden) identifying a 145 kDa subclass of intermediate filaments.

Immunoblotting: For PS1 detection in rat brain fractions, 15 μg protein in 2× sample buffer (4% SDS, 20% glycerol, 5% β-mercaptoethanol in 0.1 M Tris–HCl pH 6.8) was loaded per lane without prior heating and separated by 12% or 4–20% SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride membrane. Western blotting was performed with antisera 231-f (1:3000), R27 and R28 (both 1:1000) followed by incubation with peroxidase-conjugated anti-rabbit IgG and detection by enhanced chemiluminescence (ECL, Amersham, Freiburg). Immunoblotting with other antibodies was performed by loading 5 μg or 15 μg protein/lane with prior heating (95°C, 5 min). Samples obtained in actin fragmentation experiments and actin assembly assays were loaded with equal volumes per lane.

Isolation and subfractionation of brain cytoskeleton: Cytoskeleton was isolated from adult rat brain and subfractionated by a method of Moss [18], with minor modifications. Briefly, rat brains were homogenized in 2% Triton X-100 (v/v) in Tris/$MgCl_2$ buffer (10 mM Tris–HCl pH 7.5; 2 mM $MgCl_2$) supplemented with a cocktail of protease inhibitors (Complete, Roche Diagnostics, Mannheim). Usually a wet weight/volume ratio of 1/10 was used. The homogenate was centrifuged at 100 000 × *g* for 30 min. The detergent-insoluble pellet was homogenized in 10% sucrose (w/v) in Tris/$MgCl_2$ buffer. A discontinuous sucrose gradient consisting of 50% and 30% sucrose layers in Tris/$MgCl_2$ buffer was overlayed with the resuspended material and centrifuged at 100 000 × *g* for 3 h. The fractions were collected from the interfaces and washed in Tris/$MgCl_2$ buffer. Homogenate and fractions were dissolved in 2× SDS buffer (4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.1 M Tris–HCl pH 6.8) with Complete using a 22 gauge needle. Samples were cleared by centrifugation and their protein concentration was determined with the BioRad (Munich) DC protein assay kit.

Microfilament severing experiments: Rat brain homogenate (see above) was centrifuged at 10 000 × *g* for 30 min. The detergent-insoluble pellet was resuspended to the original volume with Tris/$MgCl_2$ buffer. The suspension was equally distributed to 2 ml Eppendorf cups and centrifuged at 10 000 × *g* for 30 min. The supernatant (wash fraction) was collected. The pellet was resuspended to the original volume with severing buffer, sheared with a 26 gauge needle and left overnight on an overhead-shaker at 4°C. For gelsolin treatment, the severing buffer consisted of Tris/$MgCl_2$ buffer supplemented with Complete plus 250 nM gelsolin and 200 μM $CaCl_2$. As a negative control, gelsolin was left out. The next day, the suspension was centrifuged at 10 000 × *g* for 30 min. The supernatant was concentrated 20-fold by precipitation with trichloroacetic acid (TCA). All samples were dissolved in 2× sample buffer.

In vitro transcription/translation: Coupled *in vitro* transcription/translation reactions were performed using the TNT T7 protocol (Promega, Mannheim) according to the manufacturer's recommodations. In brief, 50 μl reactions were performed with 1 μg of a wild-type PS1 construct in vector pSG5 at 30°C for 90 min.

Actin assembly assay: Rabbit skeletal muscle actin diluted in G-buffer (5 mM Tris–HCl pH 8.0, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM $CaCl_2$) was initially centrifuged at 100 000 × *g* for 60 min at 4°C to remove aggregated actin and small actin oligomers prior to actin assembly. A 50 μl aliquot of *in vitro* transcribed and translated wild-type PS1 was added to 130 μg purified monomeric actin in 200 μl G-buffer. Actin polymerization was initiated by increasing salt and ATP concentrations (5 mM Tris–HCl pH 8.0, 0.5 mM DTT, 1 mM ATP, 0.2 mM $CaCl_2$, 100 mM KCl, 2 mM $MgCl_2$). A 200 μl aliquot of each sample was immediately overlayed on 1.3 ml of an actin cushion (5 mM Tris–HCl pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 10% (vol/vol) glycerol) in a 1.5 ml tube. The remaining 50 μl of each sample was labeled T for 'total'. After incubation for 30 min at 25°C and subsequent centrifugation at 100 000 × *g* for 60 min at 25°C, the supernatant was removed and transferred to a fresh tube (labeled S for 'supernatant'). The resulting actin pellet was resuspended in 200 μl actin assembly buffer (labeled P for 'pellet'). Equal amounts of each fraction (T, S and P) were separated by SDS-PAGE and analyzed after Coomassie-Blue staining or by immunoblotting.

Validation of the actin assembly assay was performed using 200 μg BSA as a negative and 20 μg α-actinin as a positive control. The same set of experiments was performed with human non-muscle actin (Cytoskeleton, Denver).

RESULTS

Enrichment of alternative PS1 fragments in detergent-insoluble fractions: Initially, we observed considerable immunoreactivity for PS1 in the pellets of samples obtained by tissue extraction under comparatively mild detergent conditions and further clearance by centrifugation (data not shown). Subsequently, a detergent-insoluble pellet highly enriched in cytoskeleton was isolated from adult rat brain homogenate and fractionated by discontinuous sucrose gradient centrifugation. According to Moss [18], membrane-associated cytoskeleton is found in the 10%/30% interface fraction, whereas the 30%/50% interface contains both the cytoplasmic cytoskeleton and the membrane-associated cytoskeleton. A considerable amount of the detergent-insoluble material sedimented at the 30%/50% interface, a smaller amount at the 10%/30% interface.

The presence of PS1 protein was examined by antibody

231-f directed against residues 2–20 of the PS1 *N*-terminus and by antisera R27 and R28, both directed against residues 263–407 of the PS1 *C*-terminal loop region. PS1 immunoreactivity was found in all analyzed fractions. However, there were differences in the distribution of constitutive and alternative fragments of PS1 (Fig. 1). The constitutive 30 kDa *N*-terminal fragment was enriched in the detergent-soluble fraction, although it also appeared in the detergent-insoluble pellet. Alternative 36 kDa species were enriched in the detergent-insoluble pellet and particularly in the 30%/50% interface fraction. The constitutive 20 kDa *C*-terminal fragment was found in all fractions, but immunoreactivity was particularly high in the detergent-soluble fraction. The alternative 14 kDa species were only detected by the antibody R27 and were enriched in the detergent-insoluble pellet as well as the 30%/50% interface fraction.

These data indicate that PS1 may associate with membrane-bound or cytosolic cytoskeletal elements.

Cytoskeletal proteins are enriched in detergent-insoluble fractions: We further characterized all fractions obtained by differential centrifugation of rat brain homogenate to confirm the enrichment of cytoskeletal elements in the detergent-insoluble fractions (Fig. 1). As would be expected, actin immunoreactivity was enriched in the detergent-insoluble pellet and the 30%/50% interface fraction compared to the detergent-soluble and the 10%/30% interface fraction. Immunoreactivity of α-tubulin was high in the detergent-insoluble pellet, the 10%/30% and the 30%/50% interface fractions and low in the detergent-soluble fraction. Neurofilament immunoreactivity was only detected in detergent-insoluble fractions.

As a control, BiP immunoreactivity was analyzed in all fractions. According to its localization, this soluble protein resident in the ER lumen was only detectable in the detergent-soluble fraction (Fig. 1).

Association of PS1 fragments with microfilaments: We tried to specifically sever microfilaments in the detergent-insoluble pellet obtained from rat brain homogenate. After centrifugation, cytoskeletal fragments should appear together with their associated proteins in the supernatant. Concomitant appearance of specifically severed cytoskeleton and PS1 argues for its association with the respective type of cytoskeleton.

Non-specific fragmentation of the cytoskeleton, achieved by treating the detergent-insoluble pellet with 8 M urea overnight, led to the detection of PS1 fragments, actin, tubulin and neurofilaments in the supernatant (Fig. 2). Specific fragmentation of microfilaments was achieved with 250 nM gelsolin in the presence of calcium. After incubation with gelsolin, actin but not α-tubulin or NF-M could be detected in the supernatant. Western blotting with R27 revealed 30 kDa *N*-terminal PS1 species in supernatants that were shown to be positive for actin (Fig. 3). The antiserum R27 is directed against the entire loop region of PS1 and is capable of detecting both *N*- and *C*-terminal fragments of PS1. For confirmation of specificity, R27 was incubated with the antigen and the 30 kDa band detected by R27 was abolished by antibody preabsorption (Fig. 3).

These results indicate that at least part of the 30 kDa *N*-terminal fragment of PS1 is associated with actin filaments. However, no 36 kDa species of PS1 were solubilized by gelsolin.

Co-sedimentation of PS1 with microfilaments: To confirm the interaction of PS1 with actin filaments, we performed actin polymerization co-assembly experiments with *in vitro* transcribed and translated wild-type PS1 and purified rabbit muscle actin. Detection of PS1 in the polymerized actin pellet would further support the association of the two proteins.

Initial experiments were performed to demonstrate the specificity of this co-assembly assay. The association of α-actinin, a known actin binding protein, was investigated. As shown in Fig. 4a, α-actinin was detected in the polymerized actin pellet under conditions that promote *in vitro* actin polymerization, whereas BSA did not co-sediment in the same experiment.

Similar experiments under the same conditions were performed with *in vitro* transcribed and translated PS1. PS1 was detected in the polymerized actin pellet, and thus provided further evidence for an association of PS1 with microfilaments (Fig. 4b). PS1 also co-sedimented with human non-muscle actin, which is composed of isotypes β and γ in contrast to isotype α in skeletal muscle actin (data not shown).

DISCUSSION

In the present study, we found considerable amounts of both NTF and CTF of PS1 in detergent-insoluble fractions of rat brain homogenate. Moreover, particularly neuron-specific endoproteolytic fragments were enriched in detergent-insoluble fractions, although constitutive fragments were also present in these fractions. A previous study with mouse and human cerebral cortex demonstrated only 10% of PS1 fragments to be insoluble in Triton X-100. These fragments were shown to be associated with detergent-insoluble membranes (DIMs) but not with cytoskeletal fractions [19]. In our experiments, however, the detergent-insoluble pellet and the 30%/50% sucrose interface fraction were highly enriched in actin, tubulin and neurofilaments, each representing one of the three classes of cytoskeletal proteins microfilaments, microtubules and intermediate filaments. In the low-density 10%/30% sucrose interface fraction, which we believe to contain most of the detergent-insoluble membranes (DIMs) rich in cholesterol and glycosphingolipids, only small amounts of tubulin and PS1 were detected.

There might be the possibility that enrichment of PS1 in detergent-insoluble fractions results from the interaction with proteins other than cytoskeleton. To exclude non-cytoskeletal interaction, further experiments were performed. In microfilament fragmentation experiments, gelsolin was able to specifically sever microfilaments. In contrast to the unspecific effect of urea, the incubuation of the detergent-insoluble pellet with gelsolin and subsequent centrifugation resulted only in the detection of actin but neither tubulin nor neurofilaments in the supernatant. Together with actin, a 30 kDa NTF of PS1 was released from the pellet and detected by the antiserum R27. Thus, constitutive NTFs of PS1 are associated with microfila-

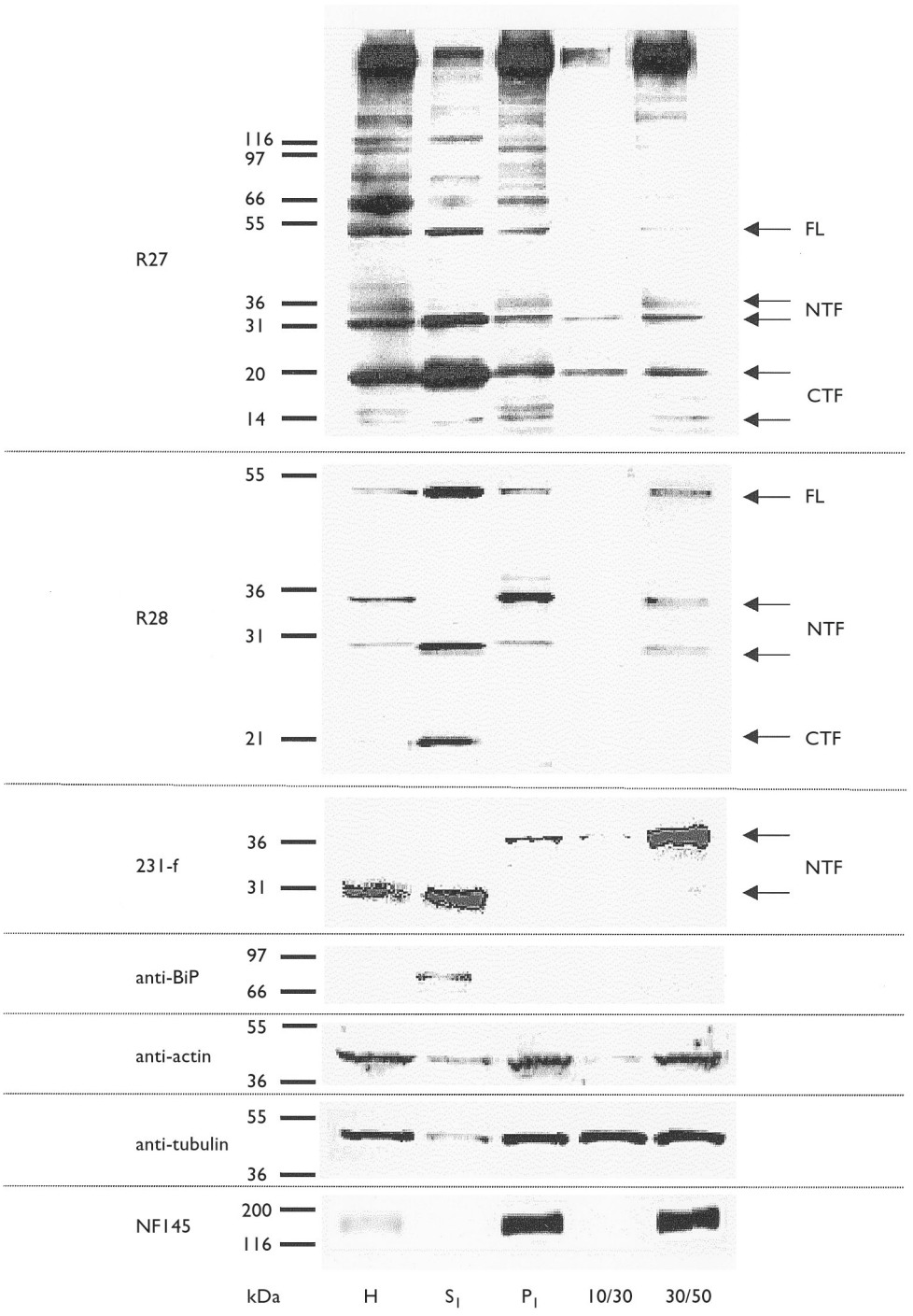

Fig. 1. Subfractionation of rat brain. Cytoskeleton was isolated from adult rat brain and subfractionated as described in the Materials and Methods section. Equal amounts of protein (5 or 15 μg) of rat brain homogenate (H), detergent-soluble supernatant (S_1), detergent-insoluble pellet (P_1) and interface fraction (10%/30% sucrose and 30%/50% sucrose) were loaded per lane. PS1 immunoreactivity (FL: full-length species at 50 kDa, NTF: N-terminal fragments at 30 kDa and 36 kDa, CTF: C-terminal fragments at 14 kDa and 20 kDa) was found in all analyzed fractions. However, there were differences in the distribution of constitutive and neuron-specific fragments of PS1. Constitutive fragments appeared predominantly in detergent-soluble fractions, whereas neuron-specific fragments were enriched in detergent-insoluble fractions.

ments. Neuron-specific fragments of PS1, that were shown to be enriched in the detergent-insoluble pellet, do not associate with actin filaments. This may indicate that these fragments specifically interact with other components of the detergent-insoluble pellet, e.g. microtubules or intermediate filaments.

Co-sedimentation of *in vitro* transcribed and translated full-length PS1 with actin filaments provided further evi-

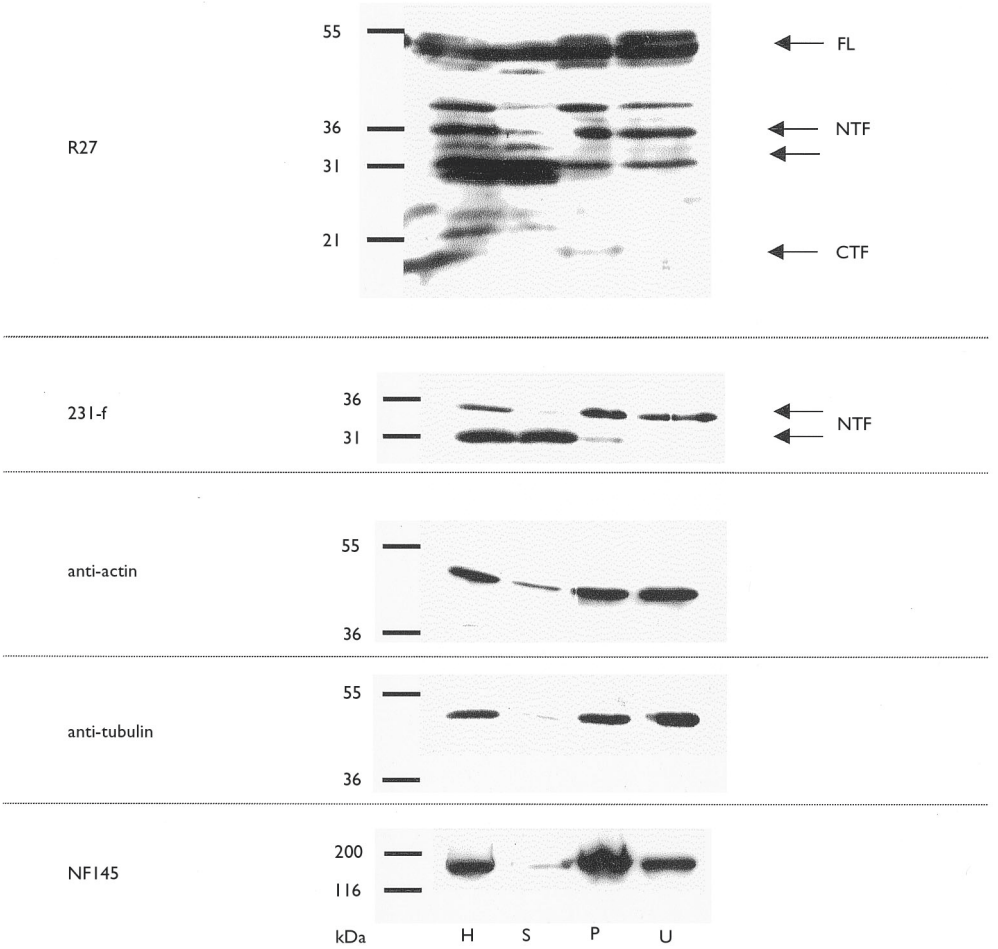

Fig. 2. Non-specific fragmentation of the cytoskeleton. Detergent-soluble (S) and -insoluble (P) fractions were prepared by centrifugation of rat brain homogenate (H) at 10 000 × **g** for 30 min. The detergent-insoluble pellet was washed with Tris/$MgCl_2$ buffer, homogenized and incubated overnight in 8 M urea in Tris/$MgCl_2$ buffer (U). Immunoblotting was performed loading equal volumes of each sample per lane. Unspecific depolymerization with urea resulted in the release of actin, tubulin and constitutive as well as neuron-specific PS1 species into the supernatant.

dence for the interaction of PS1 with actin filaments. Pigino *et al*. [20] demonstrated the association of PS1 and its fragments with microfilaments and microtubules in rat hippocampal neurons and COS cells using double-immunofluorescence.

We hypothesize that cytosolic sites of PS1 are anchored to microfilaments either directly or via some intermediary factors such as the microfilament associated proteins. This is likely to occur through the *N*-terminus or the cytosolic loop of PS1, as incubation with gelsolin released only the NTF of PS1. Potential linking proteins to microfilaments are actin-binding proteins such as filamin and FH-1 or members of the catenin/cadherin adhesion complex. The interaction of presenilins with the filamin family of actin-binding proteins was demonstrated previously [17]. A sequence analysis of PS1 revealed no obvious actin-binding motifs. Considering the diversity of the known actin-binding motifs, this does not, however, necessarily exclude an interaction of PS1 with microfilaments. Furthermore, other transmembrane proteins have been shown to associate directly or indirectly with F-actin. Occludin, a Ca^{2+}-independent intercellular adhesion molecule with four membrane-spanning segments resident in tight junctions interacts directly as well as indirectly with F-actin [21].

Recent data obtained in double-staining experiments demonstrated the co-localization of PS1 with E-cadherin, α- and β-catenin at cell–cell contacts of MDCK cells. In immunoprecipitates, PS1 fragments were found together with E-cadherin, α-, β- or γ-catenin. Furthermore, overexpression of PS1 stimulated aggregation of MDCK cells. In brain, PS1 forms complexes with both E- and N-cadherin and concentrates at synaptic adhesions [22]. In addition, fragments of PS1 are present in synaptic plasma membranes, neurite growth cone membranes, and small synaptic vesicles of rat brain [23].

The significance of the cytoskeletal association of PS1 fragments is less clear. The interaction of PS1 with the cortical microfilament network and with the catenin/cadherin adhesion complex suggests a role for PS1 in cell–cell contacts. In the context of AD, this is of particular interest for transsynaptic contacts in neurons, i.e. pathogenic PS1 mutations may disturb synaptic function. So far, this is

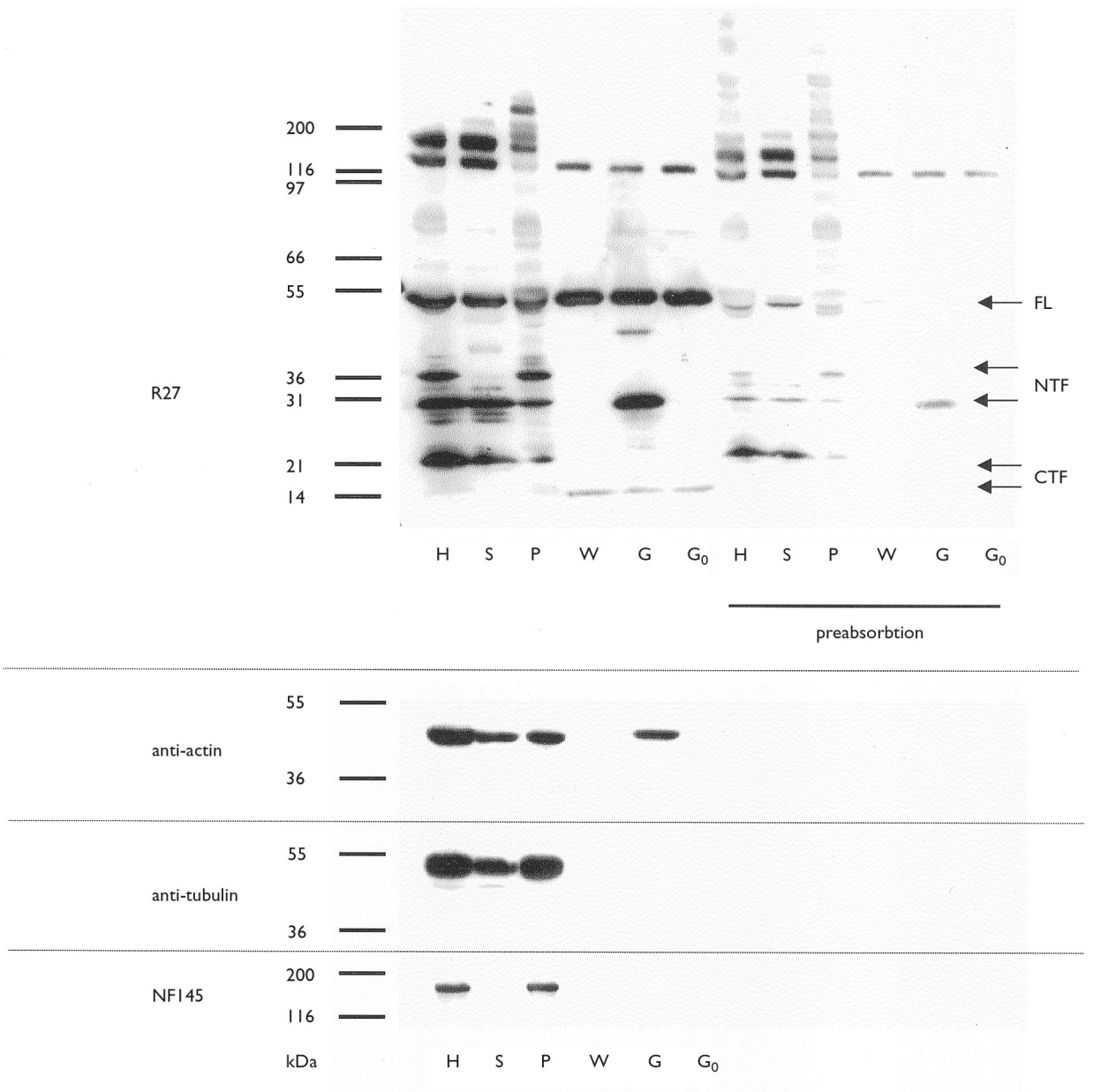

Fig. 3. Microfilament severing experiments. The experiment was similar to the experiment described in Fig. 2. The detergent-insoluble pellet was incubated in Tris/$MgCl_2$ buffer (W), 250 nM gelsolin + 200 μM $CaCl_2$ (G) and gelsolin buffer lacking gelsolin + 200 μM $CaCl_2$ (G_0). Supernatants (W, G, G_0) were concentrated 20-fold by TCA precipitation. Immunoblotting was performed by loading equal volumes of each sample per lane. Treatment of the detergent-insoluble pellet with gelsolin was shown to be specific for actin and led to the release of 30 kDa PS1 *N*-terminal fragments.

mainly attributed to the disruption of the control of intracellular calcium levels. Indeed, changes in long term potentiation have been observed in hippocampal slices from transgenic animals expressing mutant human PS1 which, as the authors suggest, might lead to altered Ca^{2+} regulation/homeostasis in the brain [24]. One could also speculate that pathogenic PS1 mutations are involved in the formation of Hirano bodies, which are mainly composed of abnormal microfilaments. They were shown to increase with advanced age, and patients with AD have significantly more Hirano bodies than normal subjects in the same age range [25].

CONCLUSION

Both constitutive and neuron-specific PS1 fragments were present in cytoskeletal fractions, suggesting an association of PS1 with members of the cytoskeleton. Although neuron-specific species of PS1 were particularly enriched, only

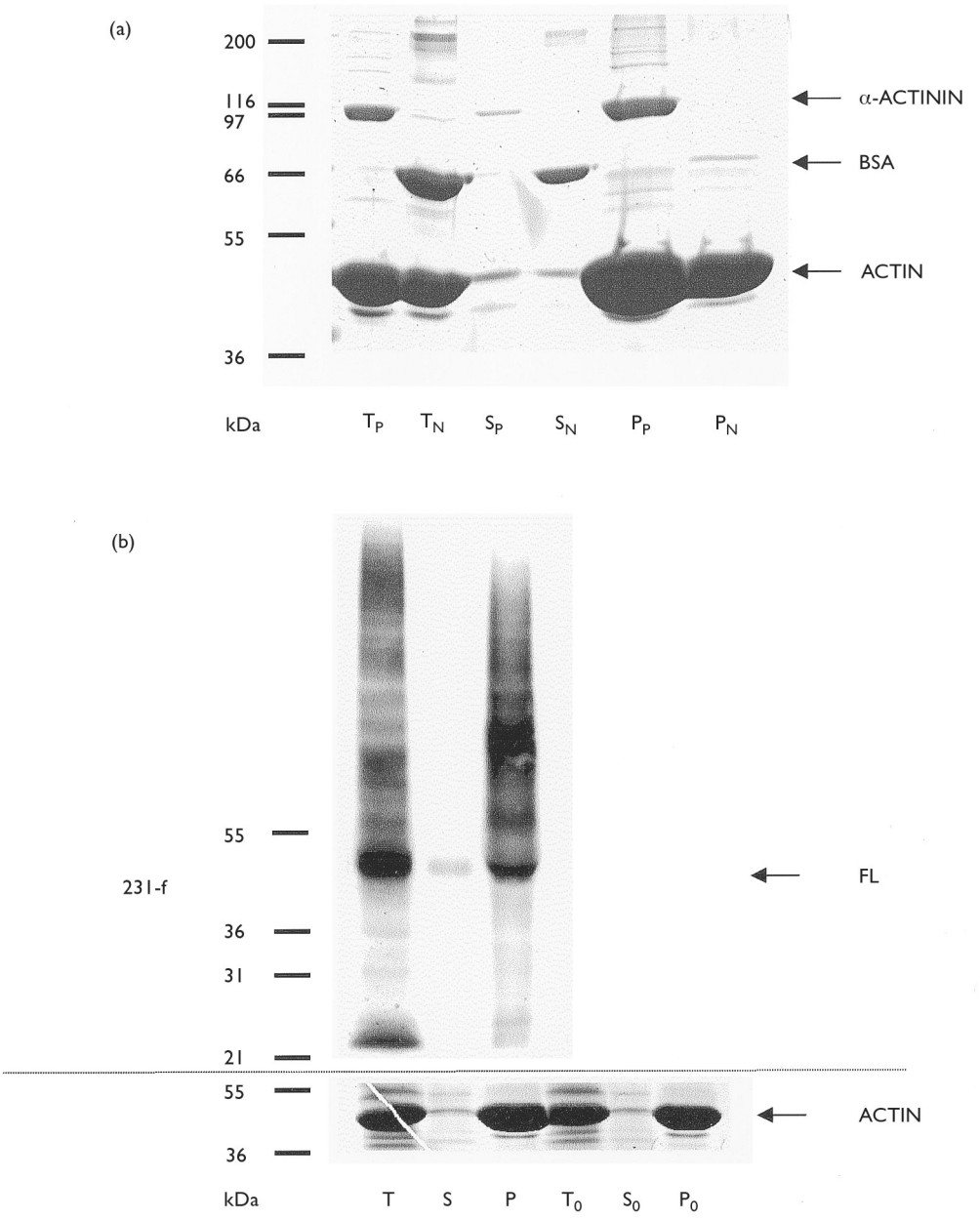

Fig. 4. PS1 co-sediments with actin filaments. (**a**) An actin assembly assay was established using 200 μg BSA as a negative control (T_N, S_N, P_N) and 20 μg α-actinin as a positive control (T_P, S_P, P_P) as described in the Materials and Methods section. Equal amounts of each fraction (T, S and P) were separated by SDS-PAGE and analyzed by Coomassie-Blue staining. Detection of a protein in the polymerized actin pellet indicates its association with microfilaments. α-Actinin was detected in the polymerized actin pellet, whereas BSA did not co-sediment in the same experiment. (**b**) Similar experiments under the same conditions demonstrated the co-sedimentation of *in vitro* transcribed and translated PS1 with microfilaments. PS1 was detected by Western blotting.

constitutive 30 kDa NTF of PS1 were shown to be associated with microfilaments. Co-sedimentation of *in vitro* translated full-length PS1 confirmed the principal interaction of PS1 with actin filaments.

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